Detection of pathogens associated with early-onset neonatal sepsis in cord blood at birth using quantitative PCR [version 1; peer review: 1 approved]

Christina W. Obiero1,2, Wilson Gumbi3, Stella Mwakio1,4, Hope Mwangudzah1, Anna C. Seale1,4,5, Mami Taniuchi6, Jie Liu6, Eric Houpt6, James A. Berkley1,7,8

1Clinical research, KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya
2Global health, Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands
3Bioscience department, KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya
4Epidemiology and Population Health, London School of Hygiene & Tropical Medicine, London, UK
5College of Health and Medical Sciences, Haramaya University, Harar, Ethiopia
6Division of Infectious Diseases and International Health, University of Virginia, Virginia, USA
7Centre for Tropical Medicine, University of Oxford, Oxford, UK
8The Childhood Acute Illness & Nutrition (CHAIN) Network, Nairobi, Kenya

First published: 05 Jan 2022, 7:3
https://doi.org/10.12688/wellcomeopenres.17386.1

Latest published: 11 May 2022, 7:3
https://doi.org/10.12688/wellcomeopenres.17386.2

Abstract

Background: Early onset neonatal sepsis (EONS) typically begins prior to, during or soon after birth and may be rapidly fatal. There is paucity of data on the aetiology of EONS in sub-Saharan Africa due to limited diagnostic capacity in this region, despite the associated significant mortality and long-term neurological impairment.

Methods: We compared pathogens detected in cord blood samples between neonates admitted to hospital with possible serious bacterial infection (pSBI) in the first 48 hours of life (cases) and neonates remaining well (controls). Cord blood was systematically collected at Kilifi County Hospital (KCH) from 2011-2016, and later tested for 21 bacterial, viral and protozoal targets using multiplex PCR via TaqMan Array Cards (TAC).

Results: Among 603 cases (101 [17%] of whom died), 179 (30%) tested positive for ≥1 target and 37 (6.1%) tested positive for multiple targets. Klebsiella oxytoca, Escherichia coli/Shigella spp., Pseudomonas aeruginosa, and Streptococcus pyogenes were commonest. Among 300 controls, 79 (26%) tested positive for ≥1 target, 11 (3.7%) were positive for multiple targets, and K. oxytoca and P. aeruginosa were most common. Cumulative odds ratios across controls: cases (survived): cases (died): E. coli/Shigella spp. 2.6 (95%CI 1.6-4.4); E. faecalis 4.0 (95%CI 1.1-15); S. agalactiae 4.5 (95%CI 1.6-13); Ureaplasma spp. 2.9 (95%CI 1.3-6.4); Enterovirus 9.1 (95%CI 2.3-37); and

Open Peer Review

Approval Status

version 2
(revision)
11 May 2022

version 1
05 Jan 2022

1. Kari A. Neemann1, University of Nebraska Medical Center, Omaha, USA

Any reports and responses or comments on the article can be found at the end of the article.
Corresponding author: Christina W. Obiero (cobiero@kemri-wellcome.org)

Author roles: Obiero CW: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Visualization, Writing - Original Draft Preparation, Writing - Review & Editing; Gumbi W: Investigation, Validation, Writing - Review & Editing; Mwakio S: Resources, Writing - Review & Editing; Mwangudzah H: Resources, Writing - Review & Editing; Seale AC: Conceptualization, Funding Acquisition, Methodology, Writing - Review & Editing; Taniuchi M: Investigation, Resources, Software, Validation, Writing - Review & Editing; Liu J: Investigation, Resources, Software, Validation, Writing - Review & Editing; Berkley JA: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Resources, Supervision, Validation, Writing - Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by Wellcome [203077; 205184, to AC]; the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) under grant OXF-TDR01; CWO is supported by the Drugs for Neglected Diseases initiative [OXF-DND02]. EH is supported in-part by the National Institutes of Health [K24 AI102972]. JAB is supported by the Bill & Melinda Gates Foundation within the Childhood Acute Illness and Nutrition (CHAIN) Network [OPP1131320] and by the MRC/DfID/Wellcome Joint Global Health Trials scheme [MR/M007367/1]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2022 Obiero CW et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Obiero CW, Gumbi W, Mwakio S et al. Detection of pathogens associated with early-onset neonatal sepsis in cord blood at birth using quantitative PCR [version 1; peer review: 1 approved] Wellcome Open Research 2022, 7:3 https://doi.org/10.12688/wellcomeopenres.17386.1

First published: 05 Jan 2022, 7:3 https://doi.org/10.12688/wellcomeopenres.17386.1
Introduction
Forty-one percent of global neonatal deaths occur in sub-Saharan Africa and the risk of dying is highest in the first week of life. Infection is a leading cause of neonatal mortality, accounting for ~37% of deaths in sub-Saharan Africa, and is associated with long-term neurological impairment. Early-onset neonatal sepsis (EONS) is often due to maternal transmission of pathogens prior to, during, or soon after birth, and can be rapidly fatal. Neonatal sepsis lacks a consensus definition and the reference point for EONS is variable, based on the timing of onset of symptoms or sampling of a positive culture, i.e., occurring within the first 48 hours, 72 hours or seven days of life. Most research conducted in developing countries has focused on culturable bacterial pathogens, with Klebsiella spp., Escherichia coli and Staphylococcus aureus identified as leading causes of EONS. Group B Streptococcus (GBS) has been variably implicated in EONS, and may be underestimated due to its rapid fatality and surveillance methodology. There are limited published data on viruses such as Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) as causes of EONS in this setting.

Blood culture is the gold standard diagnostic test for EONS, despite low sensitivity. One to two millilitres of blood volume is recommended to improve microbiogram recovery, but smaller volumes are often obtained from sick neonates. Intrapartum antimicrobials, presence of fastidious organisms and culture contamination may also contribute to low culture yields. Lack of availability of microbiology facilities, lengthy turnaround times and high rates of culture-negative sepsis contribute to antibiotic consumption, exacerbating antimicrobial resistance, affecting the gut microbiota, and potentially missing important non-culturable organisms.

Nucleic acid amplification techniques can detect a broad range of pathogens with up to 90% sensitivity and 93% specificity compared to microbial culture in some studies. Recently, a custom TaqMan Array Card (TAC) approach based on quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was applied to neonatal blood and respiratory samples in South Asia and South Africa. Causal attribution of organisms identified in blood and respiratory samples in EONS using latent class modelling was 23% in South Asia and 27% in South Africa. Bacteria were predominant and Ureaplasma spp. was identified as a significant pathogen in these studies. However, healthy controls were not sampled in identical circumstances to cases in South Asia (cases were recruited from study health facilities while controls were identified from the community using an automated algorithm; controls were older than cases at sample collection) whilst in South Africa both cases and controls were recruited from the study hospital.

Cord blood provides a potential opportunity for early pathogen detection prior to the clinical onset of infection, and with adequate sample volumes, biomarkers in cord blood may correlate with peripheral blood parameters including total and differential white blood cell counts, and acute phase reactants such as C-reactive protein, serum amyloid A, haptoglobin, interleukin-6 and procalcitonin. Culture, PCR and sequencing have identified pathogenic bacteria and correlate with acute phase reactants in cord blood. However, cord blood contamination may easily occur.

We hypothesized that pathogens detected in cord blood using a molecular technique would be associated with subsequent admission and death with suspected EONS. In a nested case control study of cord blood samples systematically collected at birth, we selected neonates hospitalized within 48 hours of life with possible serious bacterial infection (pSBI) and a random set of neonates who were sampled identically and remained well.

Materials and methods
Study design and participants
We performed a retrospective case-control study of cord blood samples obtained at delivery at Kilifi County Hospital (KCH) within a systematic clinical surveillance of maternal and neonatal adverse events (clinicaltrials.gov NCT01757028). KCH serves a mostly rural population along the Kenyan coast. About half of all admissions to the neonatal ward are from the KCH maternity department, where there are ~4000 deliveries per year. Hospital deliveries and neonatal admissions have increased since maternity user fees were abolished (Free Maternity Service policy, 2013). Maternal clinical data and cord blood samples were obtained and analysed during the surveillance (clinicaltrials.gov NCT01757028) and stored for future research. Data were collected using a standardized maternal admission record. Cord blood samples were obtained by trained clinicians using standard aseptic techniques and universal safety precautions. After delivery of the neonate and the placenta, the umbilical cord was swabbed using 70% isopropyl alcohol and spirit, double clamped and cut. Approximately 10 ml of venous cord blood was collected using either a sterile 18-gauge needle (preferred) or 5Fr gastric tube and a syringe into ethylenediamine tetra-acetic acid (EDTA) tubes (BD Diagnostics, USA), centrifuged within an hour of collection; plasma and cell pellet aliquots were then frozen separately at -80°C.

Neonates born between March 2011 and March 2016 (Figure 1) who were resident of the Kilifi Health and Demographic Surveillance System (KHDSS) and had cord blood samples available were considered for this analysis. Cases were defined as neonates hospitalized within 48 hours of life with one or more features of possible serious bacterial infection (pSBI): history of difficulty feeding, history of convulsions, movement only when stimulated, respiratory rate of ≥60 breaths/min, severe chest indrawing, and a temperature of ≥37.5°C, or ≤35.5°C. Cases were further categorized as those who died during hospitalization, and those who survived and discharged home well. Unmatched controls were randomly drawn from neonates who had cord blood samples taken in identical circumstances, did not have pSBI, were discharged home well after delivery, and survived for at least 60 days without hospitalization, determined using the KHDSS household census.
Clinical data for hospitalised neonates was systematically collected at admission using standard proforma and entered in real-time on a database within a surveillance for invasive bacterial disease\textsuperscript{38, 40}. Routine laboratory investigations for clinical care included blood culture (BACTEC Peds Plus/F bottles and BACTEC 9050 instrument, Becton Dickinson, UK) and cerebrospinal fluid (CSF) culture where indicated, as previously described\textsuperscript{41}. 

Bacillus spp., Coagulase-negative Staphylococci, Coryneforms, Micrococcus spp., and viridans Streptococci were considered clinically non-significant blood culture isolates in this context.

The Kenya Medical Research Institute Scientific Steering Committee (KEMRI SSC) approved collection of cord blood samples and clinical data (SSC 1778 and 1433). Written informed consent for collection and use of samples and data for research was obtained from all participants’ parents/guardians. This retrospective analysis was approved by the KEMRI Scientific Ethics Review Unit (SERU 3007). All studies were conducted according to relevant guidelines and regulations.

### Study size

We estimated that 600 pSBI cases and 300 controls would give 90% power and 5% alpha for minimum proportions pathogens detected in 5% of pSBI cases and 1% in healthy controls.

### Total nucleic acid extraction

Stored cord blood plasma and pellets were mixed after thawing and total nucleic acid (TNA) was extracted using the High Pure viral nucleic acid large volume kit (Roche 05114403001) following manufacturer’s instructions. For each experiment, up to 2.5 ml of the plasma/pellets mix (1.5:1) underwent a lysate preparation process, then purification and elution of 150 µl TNA using spin columns, including a High Pure Extender Assembly for large initial volume. Extrinsic controls, Phocine Herpesvirus (PhHV) and artificial construct containing the region targeted by the MS2 PCR assay were added to each sample during lysate preparation to evaluate extraction and amplification efficiency. For each batch of extractions, a blank (about 2.5 ml of nuclease-free water) was processed through the complete protocol, and later assayed to rule out any carry-over problems.

---

**Figure 1.** Organisms included in the whole blood TaqMann Array Card panel. Pathogens interrogated with TaqMan Array Card (TAC) in whole blood. The TAC is a 384-well real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) based platform consisting of 8 (shown as no. 1 to 8 above) individual microfluidic channels that can be loaded with PCR reactions containing nucleic acid extract from a clinical specimen or control material. The TAC was customised to include 21 targets (16 bacterial, 4 viral and 1 protozoal organism, tested in duplicate) and two controls (MS2 bacteriophage and phocine Herpesvirus (PhHV)). The 21 targets are shown in alphabetical order as follows: Acinetobacter baumanii, Cytomegalovirus, Enterococcus faecalis, Enterovirus, Escherichia coli/Shigella spp., Haemophilus influenzae, Herpes Simplex Virus 1, Herpes Simplex Virus 2, Klebsiella oxytoca, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium tuberculosis, Neisseria meningitidis, Plasmodium spp., Pseudomonas aeruginosa, Salmonella enterica, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, and Ureaplasma spp.
contamination during the extraction and amplification processes. A positive target in the blank would invalidate positive results for that target in the same batch of TNA extractions. Testing of TNA on TAC was done either on the same day or the day following extraction.

Detection of targets using TAC RT-qPCR
Real-time reverse transcription PCR assays were performed using a custom TAC (Thermo Fisher, CA, USA) on a QuantStudio 7 Flex instrument (Life Technologies, USA) to detect 16 bacterial, four viral and one protozoal targets (Figure 1)1,2,3). The choice of targets was based on previous studies on neonatal sepsis4,5. The uidA gene detects both E. coli and Shigella species, hence were included as a single target on the TAC cards. Primers and probes were adapted from published assays to detect acute febrile illness4,6 and sepsis7 optimized for the universal cycling conditions on the card. Positive controls were plasmids for DNA and in vitro transcripts for RNA. Cards were designed, quality-controlled, and validated at the University of Virginia who provided onsite training.

For each experiment, 25 µL of TaqMan Fast Virus one-step master mix (4444434, Applied Biosystems, Thermo Fisher Scientific) was mixed with 75 µL of TNA extract or nuclease-free water (for no template control [NTC]) to make a 100 µL PCR reaction mix. Each 100 µL PCR reaction + sample mix was then transferred into the fill port of TAC after which the TAC was then centrifuged to ensure complete filling of the reaction wells, sealed and run. The reactions included a reverse transcription at 50°C for 10 minutes, initial denaturation at 95°C for 20 seconds, then 40 three-second cycles of 95°C , and 60°C for 30 seconds. Up to eight samples were tested per card, blinded to case-control status, with one NTC included in every 10 cards to check for reagent contamination. Analysis utilized QuantStudio Real-Time PCR Software version 1.2 (Applied Biosystems, Thermo Fisher). Results were quality-checked by examining target amplification plots. Baseline adjustment for targets or reaction wells with irregular amplification was done when a false amplification curve was generated or an inaccurate threshold cycle (Ct) value was yielded. Upon review of the reaction fluorescence curves for each target, we set the cut off threshold cycle (Ct) value for all targets at <40. Samples were deemed positive when any of the duplicate reactions yielded amplification curves that crossed the threshold as defined and controls were valid. We repeated Ureaplasma spp. testing using singleplex PCR on 261 samples which had parallel positive blanks on TAC, and excluded TAC results from four samples for which repeat singleplex PCR was not possible due to depletion of TNA.

Statistical analysis
Characteristics associated with case status were investigated using backward stepwise logistic regression retaining variables with P<0.1. We initially estimated the odds ratio for all cases (survived and died) versus controls. Then, since several organisms of potential public health relevance were not detected at all in controls and could not be meaningfully analysed in this way, we estimated the cumulative odds of pSBI across ordered groups of controls: cases-survived: cases-died using ordinal logistic regression which can accommodate zero values. We tested the proportional odds assumption to confirm that the relationship between each pair of outcome variable (controls, cases-survived, and cases-died) were similar prior to performing ordinal logistic regression. We estimated the attribution fraction (AF) among cases with “punafcc” in STATA v15 (StataCorp, TX, USA)8.

Results
Of 15,409 deliveries during the study period, 604 cases and 300 controls were selected (Figure 2). One case was subsequently excluded due to sample inhibition to amplification. Thus, 603 cases comprising 502 EONS survivors and 101 EONS deaths (58 [57%] and 74 [73%] of whom died within 24 and 48 hours after birth respectively) were included. Admissions on day 0, 1 and 2 of life among EONS cases were as follows: 256 (51%), 184 (37%) and 62 (12%) respectively in 502 survivors, compared with 93 (92%), 7 (6.9%) and 1 (1.0%) in 101 deaths (P<0.001).

Compared with controls, pSBI cases were more likely to be born of mothers who were nulliparous (odds ratio [OR] 1.7, 95% confidence interval [CI] 1.2-2.3) or presented with drainage of liquor (OR 2.0, 95% CI 1.3-3.1), vaginal bleeding (OR 4.8, 95% CI 2.1-11) or oedema (OR 3.0, 95% CI 1.3-6.9) (Table 1). Admission with pSBI was not associated with maternal fever, prolonged rupture of membranes (PROM) or abnormal urinalysis at admission (prior to delivery).

Among newborns, assessment of appearance, pulse, grimace, activity, and respiration (APGAR) score <9 at 5 minutes (OR 15, 95% CI 6.2-35), resuscitation at birth (OR 3.6, 95% CI 1.8-7.3) and gestation of <32 weeks (OR 2.9, 95% CI 1.2-6.9) were associated with a pSBI case status (Table 1). Admission with pSBI was not associated with maternal fever, prolonged rupture of membranes (PROM) or abnormal urinalysis at admission (prior to delivery).

Among 502 EONS survivors and 101 EONS deaths, 141 (28%) and 38 (38%) respectively tested positive for at least one TAC target, whilst 30 (6.0%) and 7 (6.9%) tested positive for multiple targets. The most frequent organisms detected were K. oxytoca, E. coli/Shigella spp., P. aeruginosa, and S. pyogenes (Table 3). Among 300 controls, 79 (26%) tested positive for at least one target, led by K. oxytoca and P. aeruginosa, and 11 (3.7%) were positive for multiple targets. L. monocytogenes and M. tuberculosis were not detected in either cases or controls. CMV was the commonest virus detected (19/603 (3.2%) cases versus 8/300 (2.7%) controls, p=0.7) and co-detection of CMV with a bacterial target was found in four cases and two controls.

We observed four patterns of target detection by TAC (Figure 3): i) Group 1: detected in a low proportion (<5.0%) in both cases (12/603 [2.0%]) and controls (6/300 [2.0%]), (HSV 1, HSV 2, S. aureus, Salmonella enterica, S. pneumoniae, and A. baumannii);
Table 1. Maternal characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n=603)</th>
<th>Controls (n=300)</th>
<th>Univariable Odds Ratio (95% CI)*</th>
<th>P value*</th>
<th>Multivariable Odds Ratio (95% CI)*</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>26 (21-32)</td>
<td>26 (21-32)</td>
<td>1.0 (0.9 to 1.0)</td>
<td>0.593</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>60 (53-70)</td>
<td>60 (54-67)</td>
<td>1.0 (0.9 to 1.0)</td>
<td>0.632</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height, cm</td>
<td>156 (151-160)</td>
<td>156 (151-160)</td>
<td>0.9 (0.9 to 1.0)</td>
<td>0.283</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MUAC, cm</td>
<td>26 (24-28)</td>
<td>25 (24-28)</td>
<td>1.0 (0.9 to 1.1)</td>
<td>0.326</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>543 (90)</td>
<td>264 (88)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Single</td>
<td>38 (6.3)</td>
<td>24 (8.0)</td>
<td>0.8 (0.5 to 1.3)</td>
<td>0.335</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Divorced</td>
<td>2 (0.3)</td>
<td>2 (0.7)</td>
<td>0.5 (0.1 to 3.5)</td>
<td>0.472</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Widowed</td>
<td>3 (0.5)</td>
<td>1 (0.3)</td>
<td>1.5 (0.2 to 14)</td>
<td>0.744</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>17 (2.8)</td>
<td>9 (3.0)</td>
<td>0.9 (0.4 to 2.1)</td>
<td>0.839</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Education Level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>105 (17)</td>
<td>58 (19)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Primary</td>
<td>360 (60)</td>
<td>178 (59)</td>
<td>1.1 (0.8 to 1.6)</td>
<td>0.555</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Secondary</td>
<td>86 (14)</td>
<td>36 (12)</td>
<td>1.3 (0.8 to 2.2)</td>
<td>0.281</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2. Study Participant Flow. Selection of cases and controls from a cohort of 15,409 deliveries at Kilifi County Hospital (KCH) between March 2011 and March 2016. Cases were hospitalised within the first 48 hours of life, resident of the Kilifi Health Demographic Surveillance System (KHDSS) and presented with one or more of the WHO-defined criteria for possible serious bacterial infection (pSBI). Controls were resident of the KHDSS and not hospitalised within the first 60 days of life.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n=603)</th>
<th>Controls (n=300)</th>
<th>Univariable Odds Ratio (95% CI)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Multivariable Odds Ratio (95% CI)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher</td>
<td>34 (5.6)</td>
<td>12 (4.0)</td>
<td>1.6 (0.8 to 3.3)</td>
<td>0.230</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>18 (3.0)</td>
<td>16 (5.3)</td>
<td>0.6 (0.3 to 1.3)</td>
<td>0.211</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nulliparous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>383 (63)</td>
<td>207 (69)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>185 (31)</td>
<td>65 (22)</td>
<td>1.5 (1.1 to 2.1)</td>
<td>0.010</td>
<td>1.7 (1.2 to 2.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>Missing</td>
<td>35 (6.0)</td>
<td>28 (9.0)</td>
<td>0.7 (0.4 to 1.1)</td>
<td>0.143</td>
<td>0.7 (0.4 to 1.3)</td>
<td>0.304</td>
</tr>
<tr>
<td>Presenting complaints</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of fever</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>584 (97)</td>
<td>294 (98)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>10 (1.7)</td>
<td>1 (0.3)</td>
<td>5.0 (0.6 to 40)</td>
<td>0.124</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>9 (1.5)</td>
<td>5 (1.7)</td>
<td>0.9 (0.3 to 2.7)</td>
<td>0.861</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drainage of liquor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>489 (81)</td>
<td>266 (89)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>109 (18)</td>
<td>30 (10)</td>
<td>2.0 (1.3 to 3.0)</td>
<td>0.002</td>
<td>2.0 (1.3 to 3.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Missing</td>
<td>5 (0.8)</td>
<td>4 (1.3)</td>
<td>0.7 (0.2 to 2.6)</td>
<td>0.568</td>
<td>0.0 (0.0 to 0.0)</td>
<td>0.975</td>
</tr>
<tr>
<td>Ruptured membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>401 (66)</td>
<td>210 (70)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>172 (29)</td>
<td>77 (26)</td>
<td>1.2 (0.9 to 1.6)</td>
<td>0.331</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>30 (5.0)</td>
<td>13 (4.3)</td>
<td>1.2 (0.6 to 2.4)</td>
<td>0.581</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PROM &gt;18h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>471 (78)</td>
<td>240 (80)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>27 (4.5)</td>
<td>9 (3.0)</td>
<td>1.5 (0.7 to 3.3)</td>
<td>0.280</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>105 (17)</td>
<td>51 (17)</td>
<td>1.0 (0.7 to 1.5)</td>
<td>0.799</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vaginal bleeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>539 (89)</td>
<td>287 (96)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>61 (10)</td>
<td>7 (2.3)</td>
<td>4.6 (2.1 to 10)</td>
<td>&lt;0.001</td>
<td>4.8 (2.1 to 11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Missing</td>
<td>3 (1.0)</td>
<td>6 (2.0)</td>
<td>0.3 (0.1 to 1.1)</td>
<td>0.063</td>
<td>0.0 (0.0 to 0.0)</td>
<td>0.993</td>
</tr>
<tr>
<td>Dysuria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>577 (96)</td>
<td>287 (96)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>21 (3.5)</td>
<td>8 (2.7)</td>
<td>1.3 (0.6 to 3.0)</td>
<td>0.527</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>5 (0.8)</td>
<td>5 (1.7)</td>
<td>0.5 (0.1 to 1.7)</td>
<td>0.273</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Decreased foetal movements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>576 (96)</td>
<td>293 (98)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>23 (3.8)</td>
<td>1 (0.3)</td>
<td>11.7 (1.6 to 87)</td>
<td>0.016</td>
<td>6.0 (0.8 to 47)</td>
<td>0.085</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (0.7)</td>
<td>6 (2.0)</td>
<td>0.3 (0.1 to 1.2)</td>
<td>0.096</td>
<td>0.0 (0.0 to 0.0)</td>
<td>0.993</td>
</tr>
</tbody>
</table>
ii) Group 2: detected in a low proportion (<5.0%) in cases (27/603 [4.5%]) but none in controls (0/300 [0.0%]), (H. influenzae, N. meningitidis, E. faecalis, Enterovirus, Plasmodium spp., and S. agalactiae);

iii) Group 3: detected in a high proportion (≥5.0%) in both cases (123/603 [20%]) and controls (70/300 [23%]), (CMV, S. pyogenes, P. aeruginosa, and K. oxytoca); and

iv) Group 4: detected in a high proportion (≥5%) in cases (47/603 [7.8%]) and low proportion (<5.0%) in controls (6/300 [2.0%]) (K. pneumoniae, Ureaplasma spp. and E. coli/Shigella spp.).

Upon examining cumulative odds, detection of any bacterial, viral or protozoal target was not associated with pSBI and death (OR 1.3, 95% CI 1.0-1.7) (Table 3). However, the high proportions of K. oxytoca and P. aeruginosa in both cases and controls suggested contamination or clinically insignificant traces of DNA in blood. Excluding these organisms, detection of any target was associated with pSBI and death (OR 2.1, 95% CI 1.4-2.8).
Table 2. Neonatal birth characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n=603)</th>
<th>Controls (n=300)</th>
<th>Univariable Odds Ratios (95% CI)</th>
<th>P valuea</th>
<th>Multivariable Odds Ratios (95% CI)b</th>
<th>P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>2.7 (1.9-3.2)</td>
<td>3 (2.7-3.3)</td>
<td>0.4 (0.3 to 0.5)</td>
<td>&lt;0.001</td>
<td>1.2 (0.7 to 2.0)</td>
<td>0.454</td>
</tr>
<tr>
<td>Length, cm</td>
<td>47.5 (43.0-49.5)</td>
<td>48.5 (47.0-50.0)</td>
<td>0.9 (0.8 to 0.9)</td>
<td>&lt;0.001</td>
<td>1.0 (0.9 to 1.1)</td>
<td>0.814</td>
</tr>
<tr>
<td>MUAC, cm</td>
<td>10.0 (8.2-10.7)</td>
<td>10.5 (9.8-11.2)</td>
<td>0.6 (0.5 to 0.7)</td>
<td>&lt;0.001</td>
<td>0.8 (0.6 to 0.9)</td>
<td>0.010</td>
</tr>
<tr>
<td>Head circumference, cm</td>
<td>33.3 (31.0-34.9)</td>
<td>34.0 (33.0-35.0)</td>
<td>0.8 (0.8 to 0.9)</td>
<td>&lt;0.001</td>
<td>0.9 (0.8 to 0.9)</td>
<td>0.036</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>345 (57)</td>
<td>151 (50)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Female</td>
<td>258 (43)</td>
<td>149 (50)</td>
<td>0.8 (0.6 to 1.0)</td>
<td>0.051</td>
<td>0.8 (0.6 to 1.1)</td>
<td>0.177</td>
</tr>
<tr>
<td>Gestation, weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥37</td>
<td>373 (62)</td>
<td>247 (82)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>≥32 to &lt;37</td>
<td>122 (20)</td>
<td>44 (15)</td>
<td>1.8 (1.3 to 2.7)</td>
<td>0.002</td>
<td>1.2 (0.8 to 1.9)</td>
<td>0.457</td>
</tr>
<tr>
<td>&lt;32</td>
<td>88 (15)</td>
<td>6 (2.0)</td>
<td>9.7 (4.2 to 23)</td>
<td>&lt;0.001</td>
<td>2.9 (1.1 to 7.7)</td>
<td>0.035</td>
</tr>
<tr>
<td>Missing</td>
<td>20 (3.3)</td>
<td>3 (1.0)</td>
<td>4.4 (1.3 to 15)</td>
<td>0.017</td>
<td>3.0 (0.8 to 11)</td>
<td>0.096</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>441 (73)</td>
<td>205 (68)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caesarean section</td>
<td>162 (27)</td>
<td>91 (30)</td>
<td>0.8 (0.6 to 1.1)</td>
<td>0.225</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing</td>
<td>0 (0.0)</td>
<td>4 (1.3)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resuscitated at birthc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>415 (69)</td>
<td>287 (96)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>186 (31)</td>
<td>11 (4.0)</td>
<td>11.7 (6.2 to 22)</td>
<td>&lt;0.001</td>
<td>3.7 (1.8 to 7.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Missing</td>
<td>2 (0.3)</td>
<td>2 (0.7)</td>
<td>0.7 (0.1 to 4.9)</td>
<td>0.713</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>APGAR Score at 5 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥9</td>
<td>352 (58)</td>
<td>290 (97)</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>&lt;9</td>
<td>229 (38)</td>
<td>6 (2.0)</td>
<td>31.4 (14 to 72)</td>
<td>&lt;0.001</td>
<td>15 (6.2 to 35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Missing</td>
<td>22 (3.7)</td>
<td>4 (1.3)</td>
<td>4.5 (1.5 to 13)</td>
<td>0.006</td>
<td>8.8 (1.1 to 70)</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Data are N (%) or median (IQR)

Abbreviations: CI, confidence interval; kg, kilogram; cm, centimetre; MUAC, mid-upper arm circumference; APGAR, appearance, pulse, grimace, activity, and respiration.

aUnivariable logistic model for all cases vs. controls

bMultivariable logistic model for all cases vs. controls, including variables with P<0.1

E. coli/Shigella spp. (P<0.001), E. faecalis (P=0.034), S. agalactiae (P=0.004), Ureaplasma spp. (P=0.010), Enterovirus (P=0.002), and Plasmodium spp. (P=0.004) were associated with pSBI and death (Table 3). K. pneumoniae (P=0.050) and N. meningitidis (P=0.054) had P values of borderline significance.

Overall, 6.6% (95% CI 0-14) of all pSBI cases were attributed to the bacterial, viral or protozoal targets. Excluding K. oxytoca and P. aeruginosa as likely contaminants, 9.4% (95% CI 5.1-13) of cases were attributed to the tested targets. Overall, 4.5% (95% CI 0-11) and 1.6% (95% CI 0-3.9) of pSBI cases were attributed to bacterial and viral targets respectively. The leading
<table>
<thead>
<tr>
<th></th>
<th>Controls (n=300)</th>
<th>Cases (n=603)</th>
<th>Cases survived (n=502)</th>
<th>Cases died (n=101)</th>
<th>Cases vs Controls odds ratio (95% CI)</th>
<th>Cases (died) vs Cases (survived) vs Controls cumulative odds ratio (95% CI)</th>
<th>Attributable Fraction among cases (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>1 (0.3)</td>
<td>3 (0.5)</td>
<td>3 (0.6)</td>
<td>0 (0)</td>
<td>1.5 (0.2 to 14)</td>
<td>1.0 (0.3 to 3.6)</td>
<td>0</td>
</tr>
<tr>
<td>Escherichia coli/Shigella spp.</td>
<td>4 (1.3)</td>
<td>34 (5.6)</td>
<td>27 (5.4)</td>
<td>7 (6.9)</td>
<td>4.4 (1.6 to 13)</td>
<td>2.6 (1.6 to 4.4)</td>
<td>3.5 (1.7 to 5.3)</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>0 (0)</td>
<td>4 (0.7)</td>
<td>3 (0.6)</td>
<td>1 (1.0)</td>
<td>-</td>
<td>4.0 (1.1 to 15)</td>
<td>0.5 (0.0 to 1.0)</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>0 (0)</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>1 (1.0)</td>
<td>-</td>
<td>-</td>
<td>0.2 (0.0 to 0.5)</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>39 (13)</td>
<td>58 (9.6)</td>
<td>47 (9.4)</td>
<td>11 (11)</td>
<td>0.7 (0.5 to 1.1)</td>
<td>0.8 (0.5 to 1.2)</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>1 (0.3)</td>
<td>9 (1.5)</td>
<td>7 (1.4)</td>
<td>2 (2.0)</td>
<td>4.5 (0.6 to 36)</td>
<td>2.7 (1.0 to 7.3)</td>
<td>0.9 (0.0 to 1.8)</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>0 (0)</td>
<td>3 (0.5)</td>
<td>2 (0.4)</td>
<td>1 (1.0)</td>
<td>-</td>
<td>5.2 (1.0 to 28)</td>
<td>0.4 (0.0 to 0.9)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>21 (7.0)</td>
<td>32 (5.3)</td>
<td>24 (4.8)</td>
<td>8 (7.9)</td>
<td>0.7 (0.4 to 1.3)</td>
<td>0.9 (0.5 to 1.6)</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1 (0.3)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0.5 (0.0 to 8.0)</td>
<td>0.4 (0.0 to 4.9)</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>1 (0.3)</td>
<td>3 (0.5)</td>
<td>3 (0.6)</td>
<td>0 (0)</td>
<td>1.5 (0.2 to 14)</td>
<td>1.0 (0.3 to 3.6)</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>9 (3.0)</td>
<td>20 (3.3)</td>
<td>20 (4.0)</td>
<td>0 (0)</td>
<td>1.1 (0.5 to 2.5)</td>
<td>0.8 (0.5 to 1.4)</td>
<td>0</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>1 (0.3)</td>
<td>3 (0.5)</td>
<td>2 (0.4)</td>
<td>1 (1.0)</td>
<td>1.5 (0.2 to 14)</td>
<td>2.0 (0.2 to 20)</td>
<td>0.2 (0.0 to 1.0)</td>
</tr>
<tr>
<td>Ureaplasma spp.</td>
<td>2 (0.7)</td>
<td>16 (2.7)</td>
<td>12 (2.4)</td>
<td>4 (4.0)</td>
<td>4.1 (0.9 to 18)</td>
<td>2.9 (1.3 to 6.4)</td>
<td>1.7 (0.5 to 3.0)</td>
</tr>
<tr>
<td>Any bacteria</td>
<td>72 (24)</td>
<td>156 (26)</td>
<td>123 (25)</td>
<td>33 (33)</td>
<td>1.1 (0.8 to 1.5)</td>
<td>1.2 (0.9 to 1.6)</td>
<td>4.5 (0.0 to 11)</td>
</tr>
<tr>
<td>Any bacteria excluding K. oxytoca and P. aeruginosa</td>
<td>20 (6.7)</td>
<td>89 (15)</td>
<td>70 (14)</td>
<td>19 (19)</td>
<td>2.4 (1.5 to 4.0)</td>
<td>2.1 (1.5 to 3.1)</td>
<td>7.9 (4.3 to 11)</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>8 (2.7)</td>
<td>19 (3.2)</td>
<td>15 (3.0)</td>
<td>4 (4.0)</td>
<td>1.2 (0.5 to 2.7)</td>
<td>1.3 (0.6 to 2.7)</td>
<td>0.6 (0.0 to 2.7)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>0 (0)</td>
<td>6 (1.0)</td>
<td>3 (0.6)</td>
<td>3 (0.6)</td>
<td>-</td>
<td>9.1 (2.3 to 37)</td>
<td>0.9 (0.2 to 1.6)</td>
</tr>
<tr>
<td>Herpes Simplex Virus 1</td>
<td>1 (0.3)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0.5 (0.0 to 8.0)</td>
<td>0.4 (0.0 to 4.9)</td>
<td>0</td>
</tr>
<tr>
<td>Herpes Simplex Virus 2</td>
<td>1 (0.3)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0.5 (0.0 to 8.0)</td>
<td>0.4 (0.0 to 4.9)</td>
<td>0</td>
</tr>
<tr>
<td>Any viruses</td>
<td>10 (3.3)</td>
<td>27 (4.5)</td>
<td>20 (4.0)</td>
<td>7 (6.9)</td>
<td>1.4 (0.6 to 2.8)</td>
<td>1.6 (0.8 to 3.2)</td>
<td>1.6 (0.0 to 3.9)</td>
</tr>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmodium spp.</td>
<td>0 (0)</td>
<td>7 (1.2)</td>
<td>6 (1.2)</td>
<td>1 (1.0)</td>
<td>-</td>
<td>2.9 (1.4 to 6.2)</td>
<td>0.8 (0.1 to 1.4)</td>
</tr>
<tr>
<td>Any bacteria, viruses and protozoa</td>
<td>79 (26)</td>
<td>179 (30)</td>
<td>141 (28)</td>
<td>38 (38)</td>
<td>1.2 (0.9 to 1.6)</td>
<td>1.3 (1.0 to 1.7)</td>
<td>6.6 (0.0 to 14)</td>
</tr>
<tr>
<td>Any bacteria (excluding K. oxytoca and P. aeruginosa), viruses and protozoa</td>
<td>30 (10)</td>
<td>115 (19)</td>
<td>91 (18)</td>
<td>24 (24)</td>
<td>2.1 (1.4 to 3.3)</td>
<td>2.0 (1.4 to 2.8)</td>
<td>9.4 (5.1 to 13)</td>
</tr>
</tbody>
</table>

1. Ordinary logistic regression
2. Ordinal logistic regression
3. Attributable fraction is calculated from cumulative odds ratio
attributed pathogens were *E. coli/Shigella* spp. (AF 3.5%, 95% CI 1.7-5.3) and *Ureaplasma* spp. (AF 1.7, 95% CI 0.5-3.0). *E. faecalis*, *H. influenzae*, *K. pneumoniae*, *N. meningitidis*, *S. agalactiae*, *Salmonella enterica*, CMV, Enterovirus and *Plasmodium* spp. were each attributed to less than 1% of the pSBI cases.

A total of 11 (1.8%) of 603 cases had presumed pathogens isolated from blood culture at admission and *S. aureus* (n=5) was the most common isolate (Table 4). The OR for a positive admission blood culture for death among admitted pSBI cases was 1.1 (95% CI 0.24-5.2) and 0.2% (95% CI 0-3.2) of pSBI were attributed to the pathogens identified through blood culture.

**Discussion**

We used a novel approach to identify causes of EONS by investigating stored cord blood samples collected at birth with a custom TAC, spatially multiplexed PCR to interrogate the presence of multiple pathogens. This is the first study evaluating diagnostic performance using cord blood in an African setting.

These samples were obtained at delivery prior to admission with signs of pSBI. Approximately 60% of 603 EONS cases and 92% of all deaths were admitted on day 0 of life. A total of 58 of 101 (57%) deaths occurred within the first 24 hours of life, after cord blood samples had been obtained. This underscores the importance of prompt diagnosis for targeted treatment and makes a cord blood approach potentially attractive in epidemiological studies, and possibly for managing ‘at risk’ neonates since cord blood could be collected, stored and tested at a later stage if the newborn develops signs of pSBI.

*E. coli/Shigella* spp. and *Ureaplasma* spp. had the highest causal attribution in our results, supporting the latter as an important pathogen in this setting.

Figure 3. Patterns of detection of Taqmann PCR Targets. Organisms included in the TAC were detected in 4 distinct groups: Group 1 (Herpes Simplex Virus 1, Herpes Simplex Virus 2, Staphylococcus aureus, *Salmonella enterica*, *Streptococcus pneumoniae*, and *Acinetobacter baumannii*); Group 2 (Hemophilus influenzae, Neisseria meningitidis, *Enterococcus faecalis*, Enterovirus, *Plasmodium* spp., and *Streptococcus agalactiae*); Group 3 (Cytomegalovirus, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Klebsiella oxytoca*); and Group 4 (*Klebsiella pneumoniae*, *Ureaplasma* spp. and *Escherichia coli/Shigella* spp.). Weighting of cases (represented as cases/2) was done to allow for improved accuracy in assessing the distribution of organisms tested, comparing cases to controls, since cases (n=603) were ~twice more than controls (n=300). Weighting was done by calculating the number of eligible cases/controls divided by the number of enrolled cases/controls i.e. the inverse of the sampling fraction for cases/controls.
clear association of *Ureaplasma* spp. with sepsis and mortality indicates pathogenicity, asymptomatic presentation or recovery in ill neonates without targeted antimicrobials has been reported and is not unusual\(^9\). Additionally, the culture-independent molecular method identified non-culturable organisms such as Enterovirus, which is shown to cause serious sepsis-like illness in neonates in other settings\(^8\). However, despite the use of sensitive molecular assays, 90% of pSBI cases still had unknown aetiology on cord blood analysis.

The overall causal attribution of 6.6% (95% CI 0-14) increased to 9.4% (95% CI 5.1-13) with exclusion of *K. oxytoca* and *P. aeruginosa* in our study. As expected, the attributable proportion was lower than in the Sepsis Aetiology in Neonates in South Africa study (SANISA [27%, 95% CI 23-32])\(^2\) and the Aetiology of Neonatal Infection in South Asia study (ANISA [23%, 95% CI 19-26])\(^8\) since much of the latter study’s attribution went to detection of RSV in respiratory samples, which this study did not examine. Although SANISA (n=27) and ANISA (n=28) were large prospective studies and tested more targets by TAC than we did (n=21), they also failed to attribute aetiology to a large proportion of pSBI cases. There were also differences in pSBI case definitions (SANISA used a predefined set of clinical and laboratory criteria\(^2\), while ANISA used WHO clinical criteria but excluded tachypnoea\(^8\)) and differences in selection and sampling of controls (SANISA sampled healthy neonates at study hospital\(^7\) while ANISA used an automated algorithm triggered at the first postnatal visit to select randomly registered controls\(^8\)). Our pSBI definition was based on the WHO Young Infants Clinical Signs study which derived a decision rule (presence of ≥1 sign: history of difficulty feeding, history of convulsions, movement only when stimulated, respiratory rate of ≥60 breaths/min, severe chest indrawing, and a temperature of ≥37.5°C, or ≤35.5°C) predicting severe illness in neonates aged 0-6 days with 87% sensitivity and 74% specificity\(^9\). The performance of these signs in distinguishing neonates with sepsis from those without sepsis has not been adequately investigated. Current WHO\(^6\) and Kenya national paediatric guidelines\(^50\) for empiric antimicrobials in neonates suspected to have sepsis are based on this limited evidence. Neonates with sepsis often present with subtle and non-specific clinical signs that overlap with those seen in other non-infectious diagnoses\(^51\). Thus, our case definition may have resulted in the inclusion of neonates who did not have true sepsis, contributing to low attribution rates. Development and use of a highly sensitive and specific consensus definition for neonatal sepsis is critically needed in clinical practice and research\(^9\).

Bacterial organisms (25% bacterial compared to 4.1% viral targets detected) were predominant in our study, similar to SANISA\(^2\) and ANISA\(^8\) results from blood samples. Thirty percent of cord blood samples of pSBI cases in our study tested positive for at least one target by TAC, compared to blood samples in SANISA (37%)\(^2\) and ANISA (12%)\(^8\). We identified multiple targets in 7% of pSBI cases compared to 11% cases in SANISA\(^2\) and 1% in ANISA\(^8\). At least one target was positive in 28% of healthy controls in our study compared to 20% in SANISA\(^2\). Thus, background positivity of cord blood among healthy neonates in our study was greater than in SANISA. All cases and controls were first selected based on the presence or absence of pSBI. All 604 pSBI cases who resided in the KHDSS had cord blood samples available for testing and were included in this analysis. 300 controls were randomly selected from a subset of 6,295 neonates who were resident of the KHDSS, remained well during the first 60 days of life, and had cord blood samples available for testing. Therefore, we ensured that cases and controls had an equal chance of being selected in respective groups, with controls derived from

### Table 4. Admission blood culture results among cases and corresponding TAC PCR results.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Cases (n=603)</th>
<th>Blood culture</th>
<th>TAC cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presumed significant organisms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter</em> spp.</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Pantoea</em> spp.</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>5</td>
<td>1 (K. oxytoca +P. aeruginosa)</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> Group B</td>
<td>1</td>
<td>1 (CMV)</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus</em> Group G+</td>
<td>1</td>
<td>1 (K. oxytoca)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Columns show number of neonates with either positive culture or TAC (TAC organisms are indicated within the brackets).

Abbreviations: CMV, cytomegalovirus.

*These organisms were not included on the TAC.*
similar circumstances to cases for optimal group comparison, unlike in ANISA where controls were recruited from the community. Although the cases had a lower gestation age and were generally smaller than the controls based on the anthropometric measurements, we believe that bias risk was minimal since we would expect sick neonates to present with known underlying risk factors of infection, such as prematurity.

K. oxytoca and P. aeruginosa were identified in large numbers in both cases and controls. This could be due to environmental contamination of laboratory materials or reagents, which has been widely reported for K. oxytoca\(^5\), contamination of the specimen by gut flora or skin commensals post-delivery\(^3\), as was reported in SANISA\(^3\), or true subclinical detection of circulating non-viable genetic material, or low copies of organisms insufficient to cause disease. Overall causal attribution increased with exclusion of K. oxytoca and P. aeruginosa, suggesting non-significance of these bacteria in EONS. Cord blood sample contamination has been reported in studies evaluating the diagnostic use of cord blood cultures, by comparing results obtained to peripheral venous blood cultures\(^20,31,35\). Although cord blood provides a non-invasive alternative to peripheral blood sampling with better culture yields\(^3\), the risk of contamination cannot be ignored. Careful aseptic techniques and training of clinical staff are imperative to optimize sample collection and may improve the validity of results. Aseptic techniques were used during cord blood sample collection to minimise sample contamination, since identification of pathogens associated with adverse maternal and perinatal outcomes was planned\(^4\), including a recently published study on the association of flavivirus exposure with congenital microcephaly\(^5\). In addition, cord blood analysis using PCR has mostly focused on vertically transmitted viruses\(^6,8,10\), and more research on cord blood testing using molecular diagnostics is needed to better understand the clinical significance of detected organisms. Detection of organisms known to cause permanent neurodevelopmental sequelae in asymptomatic congenital infection such as CMV\(^9\) (eight healthy controls in our study) may inform management. However, we did not follow up these infants for post-discharge outcomes in this retrospective analysis. Nonetheless, the PCR detections for E. coli/Shigella spp., E. faecalis, K. pneumoniae, N. meningitidis, S. agalactiae, Ureaplasma spp., and Plasmodium spp. had clear directional association across controls, surviving cases, and cases who died.

A limitation of this study was that we could not rigorously compare cord blood PCR to cord blood culture since we did not have paired specimens. Blood culture was performed on later specimens at ward admission. Although blood culture is the gold standard test for sepsis, culture-negative neonatal sepsis is common\(^4\), and this is evident in the low positivity rate among the pSBI cases in our study. In addition, the tests differed in the volumes of blood used for processing (0.1 ml equivalent per PCR reaction versus ~2ml for culture\(^5\)) and timing of testing (immediately for culture, stored for ~5 years for TAC). Low burden of infection at the limit of detection, different sampling timepoints, and decreased S. agalactiae sampling sensitivity due to antisepsis measures associated with caesarean section delivery\(^6\), may have contributed to failure to detect S. agalactiae by cord blood TAC, in a pSBI case from whom S. agalactiae was isolated from admission blood culture five hours after delivery.

Maternal variables at delivery can aid prompt initiation of antimicrobials. Intrapartum fever (temperature ≥38°C), chorioamnionitis, pre-labour rupture of membranes ≥18 hours, preterm pre-labour rupture of membranes, PROM ≥18 hours, maternal GBS colonization or bacteriuria, multiparity, and poor intrapartum and postpartum infection control practices have previously been shown to predispose neonates to infection\(^1,6,12\). We lacked complete data on intrapartum antibiotic use and were unable to assess its impact on pathogen identification. In addition to an immunological immaturity\(^6\), prematurity, low birth weight, complicated or instrument-assisted delivery, and low APGAR scores, contribute to an increased risk of admission with EONS. Although not the primary aim of our study, we observed that being identified as very preterm (<32 weeks) as well as head circumference and MUAC, which are associated with maturity\(^6\), were associated with EONS. However, low birth weight was not associated with EONS in our study.

Although TAC provided epidemiological data on potential causes of EONS in our setting, including the role of nonculturable organisms such as Ureaplasma spp. and Enterovirus, 90% of pSBI cases lacked epidemiological attribution. The presence of presumed contaminants in both cases and controls was only discernible on a population basis rather than from an individual’s results. Thus, despite allowing for customization of a panel of pathogen targets, requirement of small blood volumes, and rapid pathogen detection, TAC in its current form may have a limited role in individual diagnosis in clinical practice, particularly in settings like ours where associated costs of setting up and using this platform will be prohibitive. Further research using this technology alongside highly specific diagnostic methods is needed to better understand the aetiology, distribution and determinants of disease. In addition, our study was limited by use of archived samples and retrospective analysis of data. Future prospective studies using specific definitions of EONS alongside paired cord blood and peripheral blood cultures are needed to better understand the performance of TAC in detection of pathogens associated with EONS.

In conclusion, we were able to identify organisms associated with subsequent EONS and death using cord blood at birth and an identically sampled comparator group of healthy neonates in sub-Saharan Africa. Further prospective research on the clinical utility of cord blood in our setting is needed alongside development and use of rapid and specific point-of-care diagnostics, that will guide prompt management in seriously ill neonates. Robust evidence of the causes of EONS is vital, given the potential for prevention and targeted treatment strategies such as maternal immunization and intrapartum antibiotic prophylaxis\(^6\), including oral azithromycin for reduction of
bacterial carriage and risk of EONS\textsuperscript{86}. Coverage for \textit{Ureaplasma} spp. in at-risk neonates should be considered when updating antimicrobial guidelines given the strength of combined data from three studies (ours, SANISA and ANISA) and the potential adverse outcomes associated with this organism\textsuperscript{87}.

**Data availability**

**Underlying data**

Harvard Dataverse: Replication Data for: Detection of pathogens associated with early-onset neonatal sepsis in cord blood at birth using quantitative PCR. [https://doi.org/10.7910/DVN/FXKGRB\textsuperscript{88}](https://doi.org/10.7910/DVN/FXKGRB)

This project contains the following underlying data:

- Maternal variables-1.tab
- Neonatal variables-1.tab
- PCR Ct values-1.tab

**Extended data**

Harvard Dataverse: Replication Data for: Detection of pathogens associated with early-onset neonatal sepsis in cord blood at birth using quantitative PCR, [https://doi.org/10.7910/DVN/FXKGRB\textsuperscript{88}](https://doi.org/10.7910/DVN/FXKGRB)

This project contains the following extended data:

- COibiero_Detection of pathogens in cord blood_Codebook.pdf
- COibiero_Detection of pathogens in cord blood_readme.txt
- Detection of pathogens at birth_Extended data.pdf

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Acknowledgements**

We thank all the children and parents/guardians who contributed to this analysis. This study is published with the permission of the Director of the KWTRP. Surveillance at Kilifi County Hospital was undertaken at the KWTRP and we thank the clinical and nursing staff and all those involved.

**References**


40. Boyer KM, Gotoff SP: Prevention of early-onset neonatal group B


50. MOH: Basic Paediatric Protocols for ages up to 5 years. Fourth ed. 2016. Reference Source


PubMed Abstract | Publisher Full Text


PubMed Abstract | Publisher Full Text | Free Full Text


PubMed Abstract | Publisher Full Text | Free Full Text

Open Peer Review

Current Peer Review Status: ✔️

Version 1

Reviewer Report 19 April 2022

https://doi.org/10.21956/wellcomeopenres.19223.r49589

© 2022 Neemann K. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Kari A. Neemann
Pediatrics, Division of Infectious diseases, Children's Hospital and Medical Center, University of Nebraska Medical Center, Omaha, NE, USA

I would like to commend the authors for preparing a well-cited and thoughtful interpretation of their results. Overall, it is hard what to make of this data in the clinical setting. None of the potential identified pathogens on the cord blood sample were identified by the traditional “gold standard” blood culture which even with its limitations is hard to account for. A large percentage of neonates in both the cases and controls had ≥1 typical pathogen identified (K. oxytoca, Streptococcus pyogenes, and P. aeruginosa); organisms which, with the noted exception for K oxytoca, are not typically considered contaminants. I agree with the authors that further prospective studies utilizing paired samples of cord blood multiplex PCR, cord blood culture, and peripheral blood cultures in EONS are needed to fully evaluate the utility of this assay in the clinical setting.

Materials and Methods:
1. “Cases were defined as neonates hospitalized within 48 hours of life with one or more features of pSBI.” Please confirm that none of the cases were discharged from the hospital and re-admitted within 48 hours of life.

2. While Staphylococcus epidermidis is a common contaminant in most populations, it can represent a true pathogen in neonates though admittedly more so as late-onset sepsis in VLBW infants. Was there any thought to including this as a pathogen?

3. Did every neonate with pSBI have a blood culture obtained? While it is mentioned as routine clinical care it is not clear whether this occurred or not. Try to determine how to interpret the positive blood culture results in the results sections.

Results:
1. ‘Table 1. Prolonged rapture of membranes’ - change rapture to rupture.

2. Figure 3. Would label on the right Groups 1-4.
Discussion:
   1. I thought the authors did a good job of describing the strengths and limitations of the study and putting this study into the context of the ANISA and SANISA trials.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Pediatric Infectious Diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Author Response 03 May 2022**

Christina Obiero, KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya

Many thanks for the comments on this manuscript. We have revised the manuscript based on reviewer’s comments and responded to specific points as below.

**Materials and Methods:**

1. “Cases were defined as neonates hospitalized within 48 hours of life with one or more features of pSBI.” Please confirm that none of the cases were discharged from the hospital and re-admitted within 48 hours of life.

Response: Thank you for this important comment. All cases included in this analysis were admitted within the first 48 hours of life. None of the pSBI cases were discharged home from the hospital and readmitted within the first 48 hours of life. Unique identifiers are used at our hospital for each admission and laboratory sample, and none of the cases had more than one
episode of admission during the first 60 days of life, including the first 48 hours of life when they
developed signs of pSBI and were hospitalised. We have clarified this in the relevant section as
shown below:

**Study design and participants, Page 6:** “None of the cases were readmitted following the
initial hospital admission, including during the first 48 hours of life.”

2. While *Staphylococcus epidermidis* is a common contaminant in most populations, it can
represent a true pathogen in neonates though admittedly more so as late-onset sepsis in
VLBW infants. Was there any thought to including this as a pathogen?

**Response:** While customising the TaqMan Array Card (TAC), we reviewed literature and considered
organisms shown to cause neonatal sepsis in previous studies as indicated on page 8 (Detection
of targets using TAC RT-qPCR). *S. epidermidis* is a common culture contaminant in different
settings including ours. It was the leading admission blood culture contaminant (n=27) among all
pSBI cases. We previously examined systematic clinical and microbiologic surveillance data from
all neonatal admissions to Kilifi County Hospital to determine association of Coagulase-negative
Staphylococci (CoNS [including *S. epidermidis*]) with case fatality and/or prolonged duration of
admission among neonates and found that CoNS were not clinically significant organisms in our
setting where long intravascular lines and invasive ventilation are not available, hence not
requiring targeted antibiotic treatment.\(^1\) In addition, our clinical setting lacks factors that often
predispose patients to colonization and invasion by CoNS e.g. use of indwelling medical devices.
We have revised the section describing the selection of TAC targets as follows:

**Detection of targets using TAC RT-qPCR, page 8:** “Organisms such as CoNS that have been
previously shown to be clinically insignificant in our setting\(^44\) were not included in the TAC
panel.”

3. Did every neonate with pSBI have a blood culture obtained? While it is mentioned as
routine clinical care it is not clear whether this occurred or not. Try to determine how to
interpret the positive blood culture results in the results sections.

**Response:** Thank you for this important comment. All neonates with pSBI had blood culture done
at admission for clinical care and as part of ongoing clinical surveillance for bacteraemia on the
ward. We have clarified this section as follows:

**Study design and participants, page 6:** “Routine laboratory investigations for all admissions
for clinical care included blood culture (BACTEC Peds Plus/F bottles and BACTEC 9050 instrument,
Becton Dickinson, UK) and cerebrospinal fluid (CSF) culture where indicated, as previously
described\(^41\).”

All 603 pSBI cases included in this analysis had blood cultures done at admission. Eleven (1.8%)
neonates had presumed pathogens (led by *Staphylococcus aureus* [n=5]) isolated from blood
culture while 37 (6.1%) had contaminants (led by *S. epidermidis* [n=27]). Cord blood samples and
admission blood culture samples were obtained at different timepoints and correlation of these
two results was not possible in our study as discussed on page 15. In the results section, we
indicated that the odds ratio for a positive admission blood culture for death among admitted
pSBI cases was 1.1 (95% CI 0.24-5.2) and 0.2% (95% CI 0-3.2) of pSBI were attributed to the pathogens identified through blood culture. This means that although bacteraemia was associated with increased mortality among pSBI cases, culture yields were low and provided little information about pathogens causing morbidity and death among these hospitalised neonates. We have updated the discussion section as follows:

Discussion, page 15: “Low aetiological attribution by culture among pSBI cases underscores the need for better diagnostics as bacteraemia was associated with an increased likelihood of case fatality.”

Results:

1. 'Table 1. Prolonged rapture of membranes' - change rapture to rupture.

Response: Thank you for this helpful observation. We have corrected the spelling as required.

2. Figure 3. Would label on the right Groups 1-4.

Response: Thank you for this helpful suggestion. We have labelled the groups on Figure 3 appropriately.

Discussion:

1. I thought the authors did a good job of describing the strengths and limitations of the study and putting this study into the context of the ANISA and SANISA trials.

Response: Many thanks for this comment. Results obtained from our study, ANISA, and SANISA demonstrate the need for more advanced diagnostics such as the use of pathogen-specific biomarkers and metagenomics to better understand the epidemiology of neonatal sepsis in low- and middle-income countries, given the large proportion of cases lacking causal attribution following use of TAC reverse-transcription quantitative polymerase chain reaction.

Reference


Competing Interests: We have no competing interests to declare.